Over-expression of *atf4* in *Xenopus* embryos interferes with neurogenesis and eye formation

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Abstract: Accumulated evidence indicates that the activating transcription factor 4 (*atf4*) is a developmentally relevant gene. Here, we report on the characterization of *atf4* in *Xenopus* embryos, which is differentially expressed in the central nervous system, eyes, blood, and the pronephros, as well as in developing endodermal organs such as the stomach, duodenum, liver, and pancreas. Ectopic expression of *atf4* in the animal hemisphere of *Xenopus* embryos had no obvious effects on the induction of neural progenitors, but suppressed neurogenesis and eye formation without promoting apoptosis. Our data suggest that tightly controlled *atf4* activities may be crucial for normal neurogenesis and early eye patterning.

Key words: *Xenopus*; *atf4*; Eye; Neurogenesis; Apoptosis

在非洲爪蛙胚胎中过表达 *atf4* 影响早期神经发育及眼睛的形成

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摘要：越来越多的证据表明转录激活因子 4(*atf*4)是一个与胚胎发育相关的基因。该文研究了非洲爪蛙 *atf4* 在胚胎发育过程中的表达和功能。atf4 特异性地表达在非洲爪蛙胚胎的脑部、眼睛、血岛、原肾、肝脏、胰腺以及胃和十二指肠的部分细胞。在非洲爪蛙胚胎的动物极半球过表达适量(不影响胚胎整体形态发生的剂量)的 atf4, 对神经上皮细胞中 sox3 的表达无明显影响, 也不引起细胞凋亡; 但是对原始神经元的标记基因以及预定形成前脑、中脑、视网膜和晶状体的前体细胞的标记基因表达都有不同程度的抑制, 最终导致无晶状体小眼的表型。该研究结果首次提示对正常的早期神经发育及眼睛形成而言, atf4 的活性需受到严格的调控。

关键词：非洲爪蛙; *atf4* 基因; 眼睛; 神经发育; 细胞凋亡

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Activating transcription factor 4 (*atf4)*, also known as CREB-2, TAXREB67, or C/ATF, is a basic leucine zipper domain transcription factor that belongs to the cAMP responsive element binding (CREB) protein family and recognizes the ATF consensus site *TGACGT*(C/A)(G/A) (Lee et al, 1987; Hai & Hartman, 2001). It is widely expressed in a variety of adult mouse and human tissues including the brain, heart, liver, spleen, thymus, lung, muscle, testis, and kidney (Tsujimoto et al, 1991; Karpinski et al, 1992; Vallejo et al, 1993). Gene targeting studies in mice revealed that *atf4* plays specific roles in the development and physiology of a number of

tissues and organs, such as lens formation, osteoclast differentiation, bone formation, synaptic plasticity, hematopoiesis, acinar cell viability, fertility, modulation of metabolic and oxidative stress, aspects of long-term memory, and lipid metabolism (Tanaka et al, 1998; Hettmann et al, 2000; Iida et al, 2007; Yang et al, 2004; Cao et al, 2010; Wang et al, 2010; Ameri & Harris, 2008). The stabilization of the *atf4* protein is required for the regulation of epithelial–mesenchymal transition of the avian neural crest (Suzuki et al, 2010). Although *atf4* is an oxidative stress- and endoplasmic reticulum stress-inducible, prodeath transcription factor in neurons (Lange et al, 2008; Galehdar et al, 2010), little is known about its role in neurogenesis during early embryonic development.

Xenopus *atf4* cDNA has previously been cloned from stage 50 gonads (Akatsuka et al, 2004; Komatsuzaki et al, 2010). To screen novel pancreatic marker genes for *Xenopus* pancreas development (Afelik et al, 2004), we identified *atf4* expressed in the developing pancreas of tadpole stage embryos. We report on the characterization of the embryonic expression pattern of *atf4* and its novel function in neurogenesis and eye formation.

1 Materials and Methods

1.1 Isolation and construction of inducible *Xenopus atf4*

Xenopus *atf4* was first identified through the screening of an adult *Xenopus* pancreas λZAP Express phage cDNA library (Afelik et al, 2004). The whole open reading frame of *Xenopus laevis atf4* was then subcloned into pGEM-T Easy vector (Promega) for generating *in situ* probes or subcloned into the pCS2+ vector (Rupp et al, 1994) containing the human glucocorticoid receptor (GR) ligand-binding domain (Gammill & Sive, 1997) for generating capped mRNA. The latter construct was designated as *atf4GR*.

1.2 Embryo manipulation and mRNA injection

One thousand human chorionic gonadotropin IUs were injected into the dorsal lymph sacs of wild-type *Xenopus laevis* adult females to induce egg spawning. Eggs were squeezed and fertilized *in vitro* with minced testes, dejellied with 2% cysteine hydrochloride (pH 7.8-8.0) 30 min after fertilization and cultured in 0.1x MBS (1.76 mmol of NaCl, 48 mmol of NaHCO$_3$, 20 mmol of KCl, 200 mmol of HEPES, 16 mmol of Mg$_2$SO$_4$, 8 mmol of CaCl$_2$, 6 mmol of Ca(NO$_3$)$_2$, and pH 7.4) buffer at room temperature. Staging of *Xenopus laevis* embryos was conducted according to Nieuwkoop and Faber

(1967). Embryos were fixed in MEMFA (0.1 mol of MOPS, pH 7.4, 2 mmol of EDTA and 4% formaldehyde) for 1 h at room temperature, washed in 100% ethanol three times for 10 min each, and stored at −20 °C in 100% ethanol. Gut dissection was carried out as reported previously (Chalmers & Slack, 1998).

To generate mRNA, *atf4GR* was linearized by NotI and transcribed with a mMESSAGE Machine Sp6 kit (Ambion). Synthesized mRNA was purified using an RNAeasy kit (Qiagen). Up to 2 ng of *atf4GR* mRNA either alone or together with 50 pg of β-*galactosidase* RNA (Chitnis et al, 1995) was injected into one blastomere of 2-cell stage embryos from the animal pole. Dexamethasone was added at stage 12.5 to activate the fusion protein (Gammill & Sive, 1997). The uninjected side served as an internal control.

1.3 Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described by Harland (1991), with modifications as reported in Hollemann et al (1999). To generate digoxigenin-labeled *atf4* antisense probes, the *atf4*-pGEM-T plasmid was linearized with SalI and transcribed with T7 RNA polymerase. The digoxigenin-labeled antisense probes for *sox3* (Penzel et al, 1997), *N-tubulin* (Oschwald et al, 1991), *pax6* (Hirsch & Harris, 1997), *otx2* (Pannese et al, 1995), *six3* (Zhou et al, 2000) and *rx1* (Mathers et al, 1997) were prepared as described in the references. The antisense probe for *pax2* was directly transcribed with the RT-PCR product containing the T7 RNA polymerase promoter. Purification of digoxigenin-labeled RNA was performed using the Qiagen (Valencia, CA) RNAeasy kit according to the RNA cleaning protocol.

1.4 TUNEL assay

Whole-mount TUNEL assay was carried out as previously reported (Hensey & Gautier, 1998). Briefly, formaldehyde fixed embryos were washed in PBS and incubated in 150 U/mL terminal deoxynucleotidyl transferase (Takara), and 0.5 mmol digoxigenin–dUTP (Roche) overnight at room temperature. The reaction was then terminated in PBS/1 mmol EDTA, at 65 °C for 2 h followed by washes in PBS. Chromogenic reaction was carried out according to Harland (1991).

1.5 RNA extraction and RT-PCR

Total RNA from freshly collected embryos was extracted using the Trizol Reagent (Invitrogen) according to the manufacturer's instructions and was then digested with DNaseI (Roche). First strand cDNA

was synthesized using Superscript I M-MLV reverse transcriptase (Invitrogen), and then subjected to PCR analysis. The annealing temperatures, cycle numbers (in parentheses), and sequences of primers used in the RT-PCR reactions are as follows: *atf4* (55℃, 24 cycles) forward 5′-*GCATGAGCCCCTCTTACTTG*-3′ and reverse 5′-*GCTTTTGGCCTGTCGAACTT*-3′, *ornithine decarboxylase (ODC)* (55℃, 23 cycles) forward 5′-*TGAATT GATGAAAGTGGCAAGG*-3′ and reverse 5′-*CAGGGCTG*

GGTTTATCACAGAT-3′.

2 Results

2.1 Spatial and temporal expression of *atf4* in *Xenopus* embryos

The *atf4* expression was hardly detectable in embryos before neurulation by whole-mount *in situ* hybridization (Fig. 1A, B). During neurulation, *atf4* was weakly expressed in the prospective midbrain,
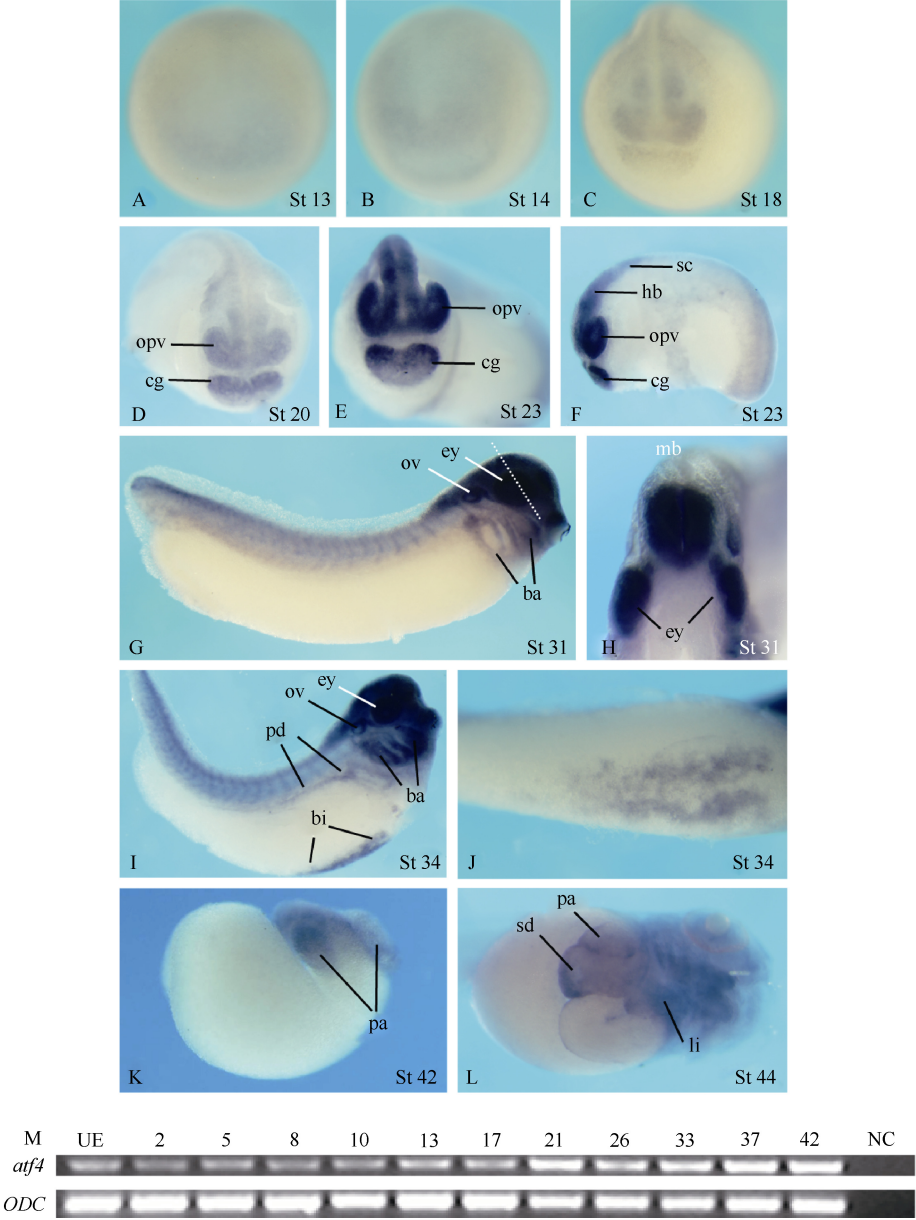

Fig. 1 Spatial and temporal expression of *atf4* in *Xenopus* embryo**s**

(A-L) Whole mount *in situ* hybridization analysis data. (A-E) Anterior views. (F, G) Lateral views. (H) Anterior view of an embryo transversally dissected at the position indicated by the white dashed line in G. (I) Lateral view. (J) Ventral view of the embryo in I showing of the expression in the blood island. (K) Isolated gut. (L) Ventral view. ba, branchial arches; bi, blood island; cg, cement gland; ey, eye; hb, hindbrain; li, liver; mb, midbrain; opv, optic vesicle; ov, otic vesicle; pa, pancreas; pd, pronephric duct; sc, spinal cord; sd, part of stomach and duodenum. (M) RT-PCR analysis reveals the temporal expression profile of *atf4* during *Xenopus* embryogenesis. UE, unfertilized egg. NC, negative control. *Ornithine decarboxylase* (*ODC*) was used as the loading control.

forebrain, and the cement gland anlage (Fig. 1C, D). At the tail bud stage, the expression levels increased and the signals in the central nervous system extended to the hindbrain and the anterior part of the neural tube. In addition, signals were also detected in the optic vesicles, branchial arches, otic vesicles, the pronephric ducts, the blood island, and the somites (Fig. 1E−J). At the transversal level of the eye and the midbrain, *atf4* expression obviously and uniformly covered the developing eyes and the whole developing neuroepithelium of the brain (Fig. 1H). At tadpole stage, *atf4* transcripts were detectable in the pancreas, the liver, and part of the stomach and duodenum (Fig. 1K, L).

Although no clear *atf4* expression signals were visualized by whole-mount *in situ* hybridization analysis in embryos before neurulation (Fig. 1A, B and data not shown), RT-PCR analysis revealed that *atf4* transcripts could be detected at all developmental stages examined, including the stages before the mid-blastula transition and unfertilized eggs (Fig.1M).

2.2 Overexpression of *atf4* inhibits neurogenesis and eye anlage formation

Atf4 knockout mice displayed microphthalmia with no recognizable lens (Tanaka et al, 1998; Hettmann et al, 2000; Masuoka & Townes, 2002), and *atf4* is specifically expressed in the eye vesicles and central nervous system of *Xenopus* embryos. We therefore investigated the effects of ectopic *atf4* activity on the morphology and expression of various molecular markers that reflect different aspects of neural development and eye formation in *Xenopus* embryos (Fig. 2). Injections of a lower dose (1 ng per embryo) of *atf4GR* mRNA led to microphthalmia (Fig. 2B) without affecting the gross morphology of the embryos, while a higher dose (2 ng per embryo) resulted in microcephaly with complete loss of eyes and slight tail deformation (Fig. 2C). We therefore chose the lower dose-injected embryos for checking marker gene expression.

Sox3 expression identifies lens placodes and proliferating neural progenitors (Penzel et al, 1997; Bourguignon et al, 1998; Bellefroid et al, 1998; Ma et al, 2007). Ectopic expression of *atf4* did not alter *sox3* expression in the neural fold, but specifically blocked its expression in the lens placode (Fig. 2D, E). With respect to primary neurons, *atf4* exerted differential effects. While the lateral stripe and the trigeminal ganglion expression of *N-tubulin* (Oschwald et al, 1991; Chitnis et al, 1995) were completely eliminated on the *atf4GR* injected side, the

medial stripe and part of the intermediate stripe were maintained (Fig. 2F, G). Overexpression of *atf4* severely inhibited *pax2* (Schlosser & Ahrens, 2004) expression in the midbrain-hindbrain boundary and completely abolished its expression in the placode, presumptive hindbrain, and spinal cord (Fig. 2H, I). Similarly, *pax6* (Hirsch & Harris, 1997) expression in the prospective hindbrain and spinal cord was completely lost and its expression in the eye anlage and presumptive forebrain and midbrain was drastically down-regulated upon *atf4* misexpression (Fig. 2J, K). Expression of other prospective lens and retinal markers like *otx2* (Pannese et al, 1995), *six3* (Zhou et al, 2000), and *rx1* (Mathers et al, 1997) were also severely inhibited on the injected side (Fig.2 L-Q). As a result, at late stages of development, a microphthalmia phenotype was observed with no lens formed and only a tiny amount of retinal and retinal pigment epithelium cells developed (Fig. 3).
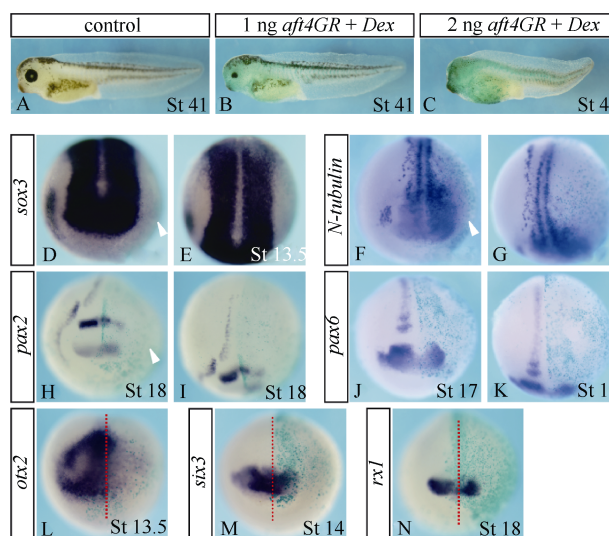

Fig. 2 Overexpression of *atf4* interferes with neurogenesis and eye anlage formation

(A-C) Lateral view of the embryos showing dose-dependent effects of atf4 on eye development. (D-N) Effects of *atf4GR* on the expression of various neural marker genes. The injected sides are on the right. Dexamethasone was added at stage 12.5. (D, F, H, J, L−N) Anterior views. (E, G, I, K) Dorsal views of D, F, H, and J, respectively. The white triangles in D, F, and H highlight the missing of marker gene expression in the lens placode, trigeminal ganglion, and placode on the injected side, respectively. The red dashed lines in L, M, and N illustrate the midline of the embryos. Dex, dexamethasone. The statistics for the phenotype are as follows: sox3 (19/20), *N-tubulin* (20/24), *pax2* (32/34), *pax6* (20/20), *otx2* (18/21), *six3* (16/18), *rx1* (12/14).

2.3 Overexpression of *atf4* in *Xenopus* embryos did not promote apoptosis

Forced expression of *atf4* promoted the death of mouse cortical neurons in culture (Lange et al, 2008; Galehdar et al, 2010). We therefore checked apoptosis in
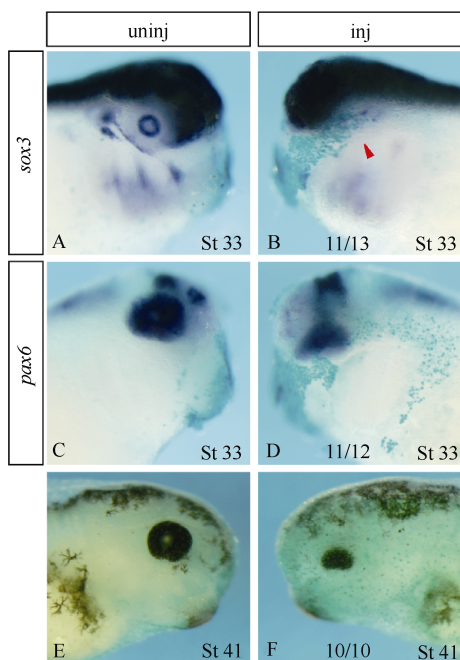
Fig. 3 Overexpression of *atf4* led to microphthalmia

(A−F) Lateral view. (A, B) The expression of sox3 in the lens is lost on the injected side labeled by red triangle. (C, D) The expression of pax6 in the retina is reduced on the injected side. (E, F) The retinal pigment epithelium is poorly developed on the injected side. uninj, uninjected side; inj, injected side.
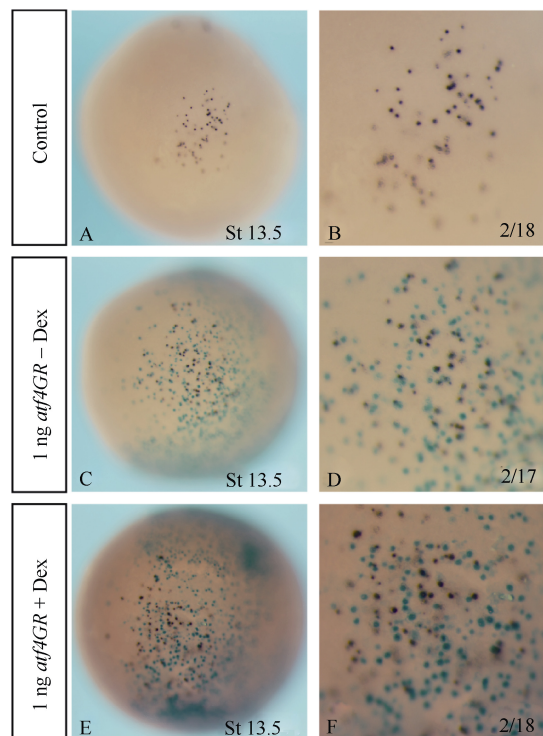

Fig. 4 Overexpression of *atf4* in *Xenopus* embryos did not cause apoptosis

At stage 13.5, the TUNEL signals detected are very weak in control embryos, as well as in atf4GR injected embryos with or without dexamethasone treatment. (A−F) Representative embryos from each group with the strongest TUNEL signals. B, D, and F are higher magnification views of A, C, and E, respectively. Statistics are indicated in B, D, and F for each group. All the rest embryos analyzed in each group showed very weak signals (data not shown). Before TUNEL assay, the embryos were subjected to beta-galactosidase staining to trace the injected sites.

atf4GR injected embryos. TUNEL staining indicated there were no obvious signal differences between the control and the injected embryos (Fig. 4), suggesting that the elimination of primary neurons and inhibition of neural gene expression upon *atf4* overexpression was not attributed to increased apoptosis.

3 Discussion

Xenopus atf4 displayed differential expression in tissues and organs derived from all three germ layers, which is consistent with data from mouse and quail studies (Murphy & Kolstø, 2000; Suzuki et al, 2010). Ectopic expression of *atf4* in *Xenopus* embryos did not affect initial neural induction, but interfered with neurogenesis by differentially blocking the primary neuron differentiation, completely inhibiting neural gene expression in the hindbrain, spinal cord, and lens placode, and partially eliminated prospective forebrain, midbrain, and retinal marker gene expression. Consequently, it led to microphthalmia with no lens formed and only a tiny amount of retinal and retinal pigment epithelium cells developed, which is reminiscent of the phenotype observed in ER81 injected embryos (Chen et al, 1999).

In mice, *Atf4* deletion did not affect the initial lens formation and the microphthalmia was caused by lens degeneration beginning at E14.5 (Tanaka et al, 1998;

Hettmann et al, 2000; Masuoka & Townes, 2002). In this study, we found that ectopic expression of *atf4* in *Xenopus* embryos blocked initial lens placode formation. *In vivo*, *atf4* is weakly expressed at early neurula stages when early neural patterning and primary neurogenesis take place. Its expression becomes strong in the nervous system and optic vesicles at the tail bud stage. Our data suggests that a tight control of the level and timing of *atf4* expression is required for proper neural development and eye formation in *Xenopus* embryos. Further studies are required to determine how *atf4* interferes with early neurogenesis and retina development. Unlike in cultured mouse neurons, overexpression of *atf4* did not promote apoptosis in *Xenopus* embryos.

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